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Establishment of Leukemic T-Cell Lines from Mice Inoculated with the MAIDS Defective Virus

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Mice inoculated with replication-competent stocks of the murine acquired immunodeficiency syndrome (MAIDS) virus are severely immunocompromised and proned to the development of T- and B-cell lymphomas. We have studied the development of T-cell lymphomas in C57BL/6 and RF/J mice inoculated with helper-free stocks of the MAIDS defective virus. We observed the expansion of T cell clones (detected by TCR gene rearrangements and by transplantation) only rarely in diseased C57BL/6 mice and slightly more frequently in RF/J mice. We succeeded in establishing four transplantable T cell tumors and malignant cell lines. The three cell lines from RF/J mice were immature T-cells (Thy-1$^+$, CD3$^-$, CD4$^+$, CD8$^+$, Mac-1$^+$), while the line from the C57BL/6 mouse had the phenotype of mature T-cells (Thy-1$^+$, CD3$^+$, CD4$^+$, CD8$^-$). All lines were virus-producers despite the fact that helper-free stocks of the virus were inoculated. These helper MuLVs most likely originated from endogenous MuLV sequences. Also, the defective viral genome was clearly detectable in one cell line and was rearranged in two other lines. These established cell lines may be useful to determine whether they share some of the characteristics of the anergic T-cells *in vivo* and to study the role of the MAIDS defective virus in T cell transformation.

INTRODUCTION

The murine acquired immunodeficiency syndrome (MAIDS), induced by a defective retrovirus (Aziz *et al.*, 1989; Chattopadhyay *et al.*, 1989), is characterized by severe dysfunctions of the T and B lymphoid cell populations (for review see (Mosier, 1986; Jolicoeur, 1991; Morse *et al.*, 1992). In mice inoculated with replication-competent viral stocks, several cell populations have been reported to be infected, including T (Kubo *et al.*, 1992; Tang *et al.*, 1992) and B (Klinken *et al.*, 1988; Chattopadhyay *et al.*, 1991; Hitoshi *et al.*, 1993) lymphocytes and macrophages (Cheung *et al.*, 1991; Bilello *et al.*, 1992).

Infection of T-cells by the MAIDS defective virus could play a role in the pathogenesis of the disease. Previous reports have indicated that infection of T-cells of mice inoculated with the replication-competent MAIDS LP-BM5 virus occurs at a relatively high frequency. Three studies (Fredrickson *et al.*, 1993; Kubo *et al.*, 1992; Tang *et al.*, 1992) reported that T-cell tumors, derived from MAIDS-diseased mice and harboring the MAIDS defective virus, could be transplanted efficiently in nude or SCID mice, respectively. In addition, an expansion of T-cells could also be detected as a clonal rearrangement of the T-cell receptor (TcR β) gene in 35% of diseased MAIDS mice inoculated with the LP-BM5 virus (Klinken *et al.*, 1988). In view of the reported transplantation studies (Fredrickson *et al.*, 1993; Kubo *et al.*, 1992; Tang *et al.*, 1992), some of these T-cell clones are likely to be malignant and to harbor a MAIDS defective viral genome.

However, the interpretation of these results is complicated by the fact that the MAIDS LP-BM5 virus stocks are rather crude and contain several strains of nondefective replication-competent helper murine leukemia viruses (MuLVs) along with the defective virus (Astier *et al.*, 1982; Guillemain *et al.*, 1980; Haas and Reshef, 1980; Legrand *et al.*, 1982; Mamoun *et al.*, 1978; Chattopadhyay *et al.*, 1989; Hartley *et al.*, 1989; Chattopadhyay *et al.*, 1991). These replication-competent MuLVs, alone or in pseudotypes with the defective virus, may infect T-cells and play a role in their transformation.

To overcome this problem and to study exclusively the cell population whose infection is required for the development of the specific immune defects seen in MAIDS, we have derived nonreplicating helper-free stocks of the MAIDS defective virus (Huang *et al.*, 1989). Interestingly, these stocks induced typical MAIDS (Huang *et al.*, 1989). We have also previously shown that, in these diseased mice, the vast majority of the infected cells belong to the B-cell lineage (Huang *et al.*, 1991). This result strongly suggested that infection of B-cells is required for the development of the immune disorders.

In the present study, we have investigated the *in vivo* T-cell transformation in mice inoculated with helper-free stocks of the MAIDS defective virus. We report the establishment of leukemic T-cell lines from these MAIDS mice. We also report that expansion and malignant transforma-

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tion of infected T-cell clones are a rare event in this system and seem to occur especially when endogenous replication-competent MuLVs, rescuing the MAIDS defective virus, are present in the inoculated mice.

MATERIALS AND METHODS

Animals and viruses

Inbred C57BL/6 and RF/J mice were purchased, respectively, from Charles River Inc. (St-Constant, Québec, Canada) and from the Jackson Laboratory (Bar Harbor, ME). Young (30–40 days old) mice were inoculated as before (Huang *et al.,* 1989, 1992), with helper-free stocks of the MAIDS defective virus, the wild-type Du5H (Huang *et al.,* 1989), the chimeric Du5H/Mo-LTR (Huang *et al.,* 1991), harboring LTR sequences from Moloney MuLV, or the tagged Du5H/neo (Huang *et al.,* 1991) and the replication-competent stocks of Du5H/Mo-LTR derived from the established T-cell line E20/34 (see below). Mice were killed between 12 and 32 weeks postinoculation (p.i.), when typical signs of advanced disease were detected, such as cervical lymphadenopathy (C57BL/6 mice) or neurological signs (RF/J).

Transplantation and cell cultivation *in vitro*

A total of 18 C57BL/6- and 13 RF/J-diseased MAIDS mice, which were inoculated at least 3 months previously with the helper-free stocks of the tagged Du5H/neo or the chimeric Du5H/Mo-LTR MAIDS defective virus, were used for transplantation. From each diseased mouse, enlarged lymph nodes and spleen (~1 g) were sampled, and a 2-ml cell suspension was prepared in RPMI-1640. The inoculum was immediately divided and injected intraperitoneally into two syngeneic recipient healthy young (<2 months old) mice, using a 23-gauge needle. The recipient mice were routinely monitored for up to 6 months. An autopsy was performed and enlarged lymphoid tissues were again collected, prepared, and reinoculated into other syngeneic recipient mice. During these steps, cell lines were established *in vitro* from diseased recipients after the first passage (T-1). Briefly, lymphoid cells were obtained from an enlarged spleen and were dispersed into RPMI-1640/10% FCS, 5×10^{-5} *M* β-mercaptoethanol, and antibiotics. The cells were seeded at a concentration of 10^6 cells/ml into five petri dishes (30 × 60 mm) for each cell line. Growing cells were split 1:2 twice a week. Lines became established after 3 to 4 weeks. Established lines were passaged for numerous months.

Cell surface labeling and cell sorting

Cells from established cell lines were labeled with antibodies. A description of the antibodies has been given elsewhere (Paquette *et al.,* 1992). Directly coupled cell surface markers monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated 30H12 (murine anti-Thy 1.2), FITC-145-2C11 (murine anti-CD3), FITC-GK 1.5 (murine anti-CD4), and FITC-ly2 (murine anti-CD8) were obtained from Dr. P. R. Sekaly (Clinical Research Institute, Montreal, Québec). The FITC-MRC OX-7 (murine anti-Thy 1.1) and the FITC-murine anti-μ were obtained, respectively, from Cedarlane Laboratories (Hornby, Ontario, Canada), and Kirkeengaard & Perry Laboratory, Inc. (MD). Cell surface markers HB58 (187-1) (murine anti-C_κ) were obtained from the American Type Culture Collection (MD). The Ra36B2 (murine anti-B220) monoclonal antibody was a kind gift of R. Coffman, DNAX Research Institute of Cellular and Molecular Biology (Palo Alto, CA). The Anti-Mac-1 antibody was from Boehringer-Mannheim Inc. (Montréal, Québec). The anti-Rat IgG-FITC (mouse adsorbed, Kirkeengaard & Perry, Inc.) was used as a second antibody (for C_κ, B220, and Mac-1). Cell lines were analyzed by fluorescence-activated cell sorting (FACS) with a FACScan (Becton–Dickinson).

Probes

The D30 (Aziz *et al.,* 1989), Moloney MuLV U3 LTR (Savard *et al.,* 1987; Poirier and Jolicoeur, 1989), neo (Huang *et al.,* 1991) ecotropic MuLV (Chattopadhyay *et al.,* 1980), (Ig) JH (Poirier and Jolicoeur, 1989; Alt *et al.,* 1981), C_κ (Poirier and Jolicoeur, 1989; Lewis *et al.,* 1982; Max *et al.,* 1981), and the β T-cell receptor RBL5 (Poirier and Jolicoeur, 1989; Caccia *et al.,* 1984) probes have been described elsewhere. For Southern blot hybridization, DNA fragments were ^{32}P-labeled as before (Poirier and Jolicoeur, 1989; Feinberg and Vogelstein, 1983).

DNA extraction and hybridization

Cellular DNA was prepared, digested with restriction endonucleases, and hybridized by the Southern technique, essentially as described previously (Huang *et al.,* 1989; Poirier and Jolicoeur, 1989).

RNA extraction and hybridization

Total RNA was extracted (Chomczynski and Sacchi, 1987) and hybridized with a ^{32}P-labeled D30 probe as previously described (Huang *et al.,* 1992).

Labeling of cells and immunoprecipitation

Immunoprecipitation of [^{35}S]methionine (100 μCi/ml)-labeled cell extract was carried out with goat anti-p30 polyclonal antibodies, as described previously (Huang and Jolicoeur, 1990).

Reverse transcriptase (RT) activity

The reverse transcriptase activity was performed on supernatants of cell lines, as described previously (Huang *et al.,* 1989; Gorska-Flipot *et al.,* 1992).

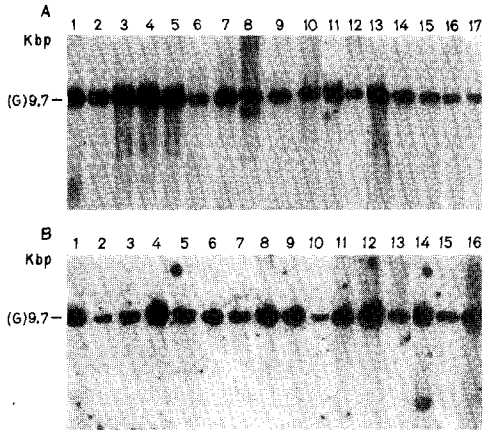

FIG. 1. Southern blot analysis of TcR gene rearrangements in organs of MAIDS mice. DNAs from diseased spleen or lymph nodes of representative mice inoculated with the MAIDS defective Du5H, Du5H/Mo-LTR, or Du5H/neo viruses, were digested with *Hin*dIII and processed by the Southern technique for hybridization with the ^{32}P-labeled cell T receptor (TcR-β) RBL5 probe. DNAs are from mice of the C57BL/6 (A) or the RF/J (B) strain. As controls, DNAs from the spleen of uninjected C57BL/6 (A, lane 17) or RF/J (B, lane 16) mice are shown. Note that TcR-β clonal gene rearrangements were observed for one C57BL/6 (A, lane 8) and one RF/J (B, lane 14) mouse. G, germ-line; Numbers, fragment length in kbp.

RESULTS

Rare clonal T-cell expansion in diseased MAIDS mice

When nonreplicating helper-free stocks of the MAIDS defective virus are inoculated into mice, most infected cells belong to the B-cell lineage, and T-cells are rarely infected by the defective virus (Huang *et al.*, 1991; Simard *et al.*, 1994). However, after inoculation of replication-competent stocks of the uncloned LP-BM5 MAIDS virus, T-cell lymphomas have been reported to occur at a relatively high frequency (Klinken *et al.*, 1988; Kubo *et al.*, 1992; Tang *et al.*, 1992). We investigated the frequency at which T-cell lymphomas develop in 36 C57BL/6- and 35 RF/J-diseased MAIDS mice inoculated with helper-free stocks of the molecularly cloned MAIDS defective virus (Du5H) and in 16 C57BL/6 mice inoculated with replication-competent stocks of the molecularly cloned MAIDS defective virus.

We initially analyzed the clonal rearrangement of the T-cell receptor β gene (TcR β), in enlarged organs of these mice (sacrificed between 12 and 32 weeks p.i.), as an indication of clonal growth of T-cell populations. Analysis by Southern blots was performed on *Hin*dIII-digested DNAs, using the TcR β-specific RBL5 probe. Clonal TcR β gene rearrangements were observed in 1 of 36 (2.8%) C57BL/6 mice and 1 of 35 (2.8%) RF/J mice inoculated with the helper-free stocks of the MAIDS defective virus (Fig. 1). None of the 16 C57BL/6 mice (<6.2%) inoculated with the replication-competent stocks of the MAIDS virus showed a clonal TcR β gene rearrangement (data not shown).

These results indicated that a T-cell expansion, reflected by a detectable TcR β gene rearrangement, is much less frequent than what has been reported with the LP-BM5 virus (Klinken *et al.*, 1988), when a molecularly cloned MAIDS defective virus (pseudotyped with helper MuLV or as helper-free stocks) is used for inoculation.

Malignant T-cell lymphomas derived from diseased MAIDS mice

Since some T-cell clones, possibly malignant, were detected in about 2.8% of mice inoculated with our stocks of the MAIDS virus, other T-cell clones, too small to be detectable by this technique, may also be present in these organs. We used a possibly more sensitive technique, the *in vivo* transplantation technique, to detect some of the T-cells which may be malignant.

Lymphoid cells, obtained directly from the enlarged spleens and/or lymph nodes of MAIDS mice, were transplanted intraperitoneally into healthy syngeneic recipients. These mice were observed routinely for up to 6 months for the detection of signs of disease. The grafted cells from 1 of 18 (5.6%) C57BL/6 MAIDS mice (E2034) and 3 of 13 (23.1%) RF/J MAIDS mice (E2028, CS016, CS031) were able to induce a proliferative disease in the syngeneic recipient mice after a short latency of approximately 2 weeks. These transplanted mice showed splenomegaly, lymphadenopathy, and liver and kidney infiltration. Cells from the enlarged organs of these first transplanted recipient mice (T-1) were reimplanted into healthy recipients (T-2), which also became diseased shortly after the transplantation. The transplantation procedure could be maintained for numerous passages *in vivo* (T-3, T-4). All the other C57BL/6 recipient and noninoculated mice (n = 10) remained healthy during the observation period (6 months). At autopsy, no lesion was observed in all these mice.

On the other hand, all the other transplanted RF/J recipients (T-1 and T-2), but not the noninoculated RF/J mice (n = 10), developed a slow but progressive disease typical of MAIDS. The latency indicated that the splenomegaly and the lymphadenopathy were unlikely caused by the growth of lymphoma cells from grafted cells, but were rather the result of transmission of the virus produced by the transplanted cells. Indeed, using a probe specific for ecotropic MuLV (Chattopadhyay *et al.*, 1980), we observed that ecotropic MuLV RNAs were expressed in all diseased RF/J mice tested (Fig. 2A, lanes 1–8), but not in diseased C57BL/6 mice (Fig. 2A, lanes 9–13). Although helper-free stocks of the virus were used, the MAIDS defective virus was almost always rescued by endogenous helper MuLVs in the RF/J mice.

Establishment of malignant T-cell lines from transplantable tumors from diseased MAIDS mice and their molecular characterization

To facilitate the characterization of the four distinct transplantable tumors obtained *in vivo,* they were estab-

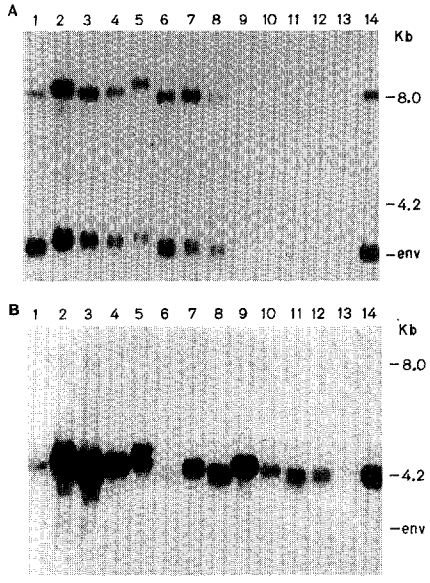

FIG. 2. Northern blot analysis of endogenous ecotropic MuLV in organs of MAIDS mice. Total RNA was extracted from diseased spleens or lymph nodes of RF/J (lanes 1–8) or C57BL/6 (lanes 9–13) mice inoculated with the helper-free stocks of the Du5H-Mo/LTR defective virus. As a positive control, spleen RNA from a C57BL/6 mouse was inoculated with the MAIDS defective virus rescued with the replication-competent B-tropic G6T2 helper MuLV (lane 14). RNAs were detected with the eco probe (A) or with the MAIDS-specific D30 probe (B).

lished *in vitro* as cell lines using cells of the recipient T-1 mice. To insure that the transplanted tumors and their corresponding cell lines were effectively derived from the original tumors, we performed a Southern blot analysis on cellular DNA extracted from them, using the TcR β, C_κ and the JH probes to detect gene rearrangements specific to T- or B-cells, respectively. A common clonal TcR β gene rearrangement was effectively observed in DNA from tumors (donor and recipients) and cell lines E2034 and CS016 (Fig. 3A), indicating that the original T-cell expansion seen in the donor mouse was malignant. In tumors (donor and recipients) and cell lines E2028 and CS031, common clonal JH rearrangements were also observed (Fig. 3B), but no clonal TcR β gene rearrangement was apparent (data not shown). In addition, no clonal C_κ rearrangement was detected in all the transplanted tumors and cell lines (data not shown). These data confirmed that the four tumors isolated from the recipient mice and the cell lines established from them were from an expanded clone detectable in the MAIDS donor mice.

To determine whether MAIDS defective proviruses were integrated into these transplantable tumors and established cell lines, we tried to visualize newly acquired virus–cell junction fragments. DNAs were digested with *Eco*RI and analyzed by the Southern blot procedure using the virus-specific U3 LTR probe (for tumors and cell lines E2028 and E2034 derived from mice inoculated with Du5H/Mo-LTR) or the neo probe (for tumors and cell lines CS016 and CS031 derived from mice inoculated with Du5H/Neo). In tumors and cell line E2034, common proviruses were present in all samples analyzed, although noncommon fragments were also detected (Fig. 4A). In tumors and cell line E2028, a common pattern of proviruses was detected between all the subpassages analyzed, but this pattern was not detected in the original donor tissue (Fig. 4B). In tumor and cell lines CS016 and CS031, newly acquired viral fragment could not be detected with the neo probe (data not shown), suggesting that either these tumor cells did not harbor the MAIDS defective viral genome or that the defective viral genome of the inoculated virus was rearranged and had lost the neo gene. A result consistent with this later hypothesis was that newly acquired common provirus could be detected in tumors and cell line CS016 (Fig. 4C), using the D30 probe specific for the MAIDS virus (Aziz *et al.*, 1989; Huang *et al.*, 1989), but not in tumors and cells line CS031 (data not shown).

For all four tumors and cell lines, the presence of newly acquired proviruses was also assessed by digesting the cellular DNAs with *Pst*I, an enzyme which generates a 4.2-kbp internal fragment after cleaving within both LTRs

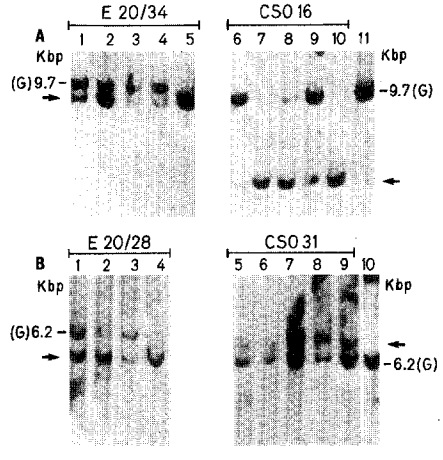

FIG. 3. Southern blot analysis of Ig and TcR gene rearrangements in transplantable T cell tumors and established T cell lines from MAIDS mice. DNAs were extracted from enlarged lymphoid organs (spleen, lymph nodes) of MAIDS donor mice and from the T-1 and T-2 transplantable tumors derived from these infiltrated organs. DNAs were digested with *Hin*dIII (A) or *Eco*RI (B) and hybridized with ^{32}P-labeled T cell receptor (TcR-β) RBL5 (A) or (Ig) J_H (B) probe. G, germ line; Numbers, fragment length in kbp. (A) DNAs from the spleen (lanes 1 and 6), lymph node (lane 2) of the initial donor E20/34 (lanes 1–5), or CS016 (lanes 6–10) MAIDS mouse, from the T-1 (lanes 3 and 7), T-2 (lanes 4, 8, and 9) transplantable T cell tumors, as well as from the established cell line (lanes 5 and 10). Lane 11 represents DNA from a normal mouse. Arrows represent common TcR clonal gene rearrangement. (B) DNAs from the spleen (lanes 1 and 5), lymph node (lane 6) of the initial donor E20/28 (lanes 1–4), or CS031 (lanes 5–9) MAIDS mouse, from the T-1 (lanes 2 and 7), T-2 (lanes 3 and 8) transplantable T cell tumors, as well as from established cell line (lanes 4 and 9). Lane 10 represents DNA from a normal mouse. Arrows represent common J_H clonal gene rearrangement.

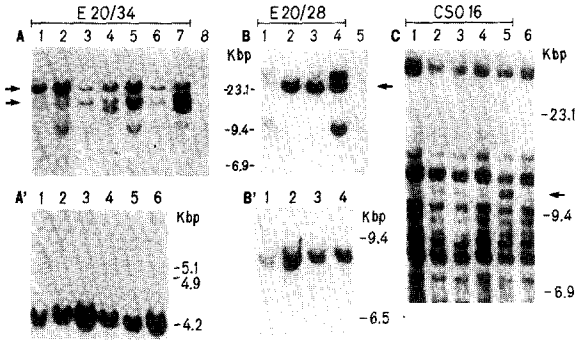

FIG. 4. Southern blot analysis of provirus integration in transplantable T cell tumors and established T cell lines from MAIDS mice. The same DNAs shown in Fig. 3 were digested with *Eco*RI (A and B), *Sac*I (C), or *Pst*I (A′ and B′) and hybridized with ^{32}P-labeled Moloney U3 LTR (A, A′, B, B′) or D30 (C) probe. (A and A′) DNAs from the spleen (lane 1), lymph node (lane 2) of the initial donor mouse E20/34, from the T-1 (lanes 3 and 4), T-2 (lanes 5 and 6) transplantable T cell tumors, as well as from the established cell line E20/34 (lane 7). Lane 8 represents DNA from a normal C57BL/6 mouse. (B and B′) DNAs from the spleen (lane 1) of the initial donor mouse E20/28, from the T-1 (lane 2), T-2 (lane 3) transplantable T cell tumors, as well as from the established cell line E20/28 (lane 4). Lane 5 represents DNA from a normal RF/J mouse. (C) DNAs from the spleen of the initial donor mouse CS016 (lane 1), from the T-1 (lane 2) and T-2 (lanes 3 and 4) transplantable T cell tumors, as well as from the established cell line CS016 (lane 5). Lane 6 represents DNA from a normal RF/J mouse. Arrows show common newly acquired virus-cell junction fragments for each line.

of the MAIDS defective viral genome (Huang *et al.*, 1989). Hybridization was done with the Moloney MuLV-specific U3 LTR probe or the D30 probe. The expected 4.2-kbp fragment was detected in tumors and cell line E2034 DNA (Fig. 4A′, lanes 1 to 6), but not in the other tumors. In tumors and cell line E2028 DNA, an unexpected full-length provirus of around 8 kbp was detected (Fig. 4B′, lanes 1 to 4). In the other tumors and cell lines CS016 and CS031, no internal 4.2-kbp *Pst*I fragment could be detected with the D30 probe (data not shown). In these two tumors, it was neither possible to detect a full-length 8-kbp provirus, most probably because of the high background obtained on cellular DNA with this probe in this size range (data not shown). A summary of the data obtained by Southern blot analysis of these four tumors and cell lines is presented in Table 1. It appears that all four transplantable tumors and cell lines were derived from a clone already detected in the original donor enlarged organ.

The malignant T-cells derived from diseased MAIDS mice are virus producers

To further characterize these lines, Northern blot analysis was performed with the D30 probe, specific for the MAIDS defective virus (Aziz *et al.*, 1989; Huang *et al.*, 1989). The expected 4.2-kb MAIDS defective virus RNA, in addition to other RNA species was revealed in E2034 cells (data not shown). However, in the other three cell lines, which were derived from RF/J mice, no 4.2-kb RNA species was detected and an apparently full-length 8-kb RNA was observed (Table 2). These RNA species may represent aberrant transcripts from the MAIDS defective virus since the D30 probe was not detecting endogenous MuLV RNAs in most of the RF/J mouse organs in which these aberrant transcripts were shown to be present (Fig. 2, compare A and B). Moreover, the D30 probe did not detect the full-length (8.0 kb) and the env-specific RNAs of the B-tropic ecotropic G6T2 helper MuLV used as a positive control (Huang *et al.*, 1989) (Fig. 2B, lane 14).

To analyze the viral proteins in these cell lines, we used the immunoprecipitation technique with the goat anti-p30 antibodies. In E2034 cells, both the Pr60^{gag}, encoded by the MAIDS defective virus (Huang and Jolicoeur, 1990), and the Pr65^{gag} encoded by helper MuLV, were clearly detected (Fig. 5, lane 1), indicating that the MAIDS defective virus inoculated as a helper-free stock was rescued by endogenous helper MuLV in this mouse cell line. In the E2028 RF/J cell line, a Pr60^{gag}-related species, possibly slightly longer, was detected (Fig. 5, lane 2), but no evidence of the Pr60^{gag} was found in lines

TABLE 1

CHARACTERISTICS OF THE TRANSPLANTABLE TUMORS AND ESTABLISHED CELL LINES DERIVED FROM DISEASED C57BL/6 OR RF/J MICE INJECTED WITH HELPER-FREE STOCKS OF THE MAIDS DEFECTIVE VIRUS

Tumors and cell lines	Mouse strain	Defective virus[a]	Latency of disease induction (days)[b] (tumors)	Rearrangement[c] TcR	J_H	C_κ	Clonal provirus integration	Provirus length
E20/34	C57BL/67	U3M	13	+	−	−	+	4.2
E20/28	RF/J	U3M	10	−	+	−	+[d]	8
CS016	RF/J	NEO	14	+	−	−	+	N.D.
CS031	RF/J	NEO	13	−	+	−	N.D.[e]	N.D.

[a] Helper-free chimeric Du5H/Mo-LTR (U3M) or tagged Du5H/Neo defective MAIDS viruses were inoculated.
[b] Time (in days) at which recipient T-1 mice were moribound after receiving 0.5 g of enlarged lymphoid tissues.
[c] Rearrangement found in transplantable tumors.
[d] Not found in donor DNA, but detected in transplanted tumors and cell lines.
[e] N.D., not detected.

TABLE 2

CHARACTERISTICS OF THE CELL LINES ESTABLISHED *in Vitro* FROM TRANSPLANTABLE TUMORS OF C57BL/6 OR RF/J MICE INOCULATED WITH THE MAIDS VIRUS

Cell line[a]	Mouse strain	Clonality between tumor and cell line[b]	RNA detected (kb)	sRT[c]	Viral protein detected[d]
E20/34	C57BL/6	+	4.2, ~3.5, ~8	+++	Pr60, Pr65
E20/28	RF/J	+	~8	+	~Pr60, Pr65
CS016	RF/J	+	~8	+	Pr65
CS031	RF/J	+	~8	+	Pr65

[a] All lines were derived from tumors after their first transplantation (T1).
[b] Clonality was measured by TcR, J_H, or C_κ rearrangements or by provirus integration.
[c] RT, reverse transcriptase assay performed on medium of cells in culture.
[d] The precursor $Pr65^{gag}$ is encoded by helper MuLV while the $Pr60^{gag}$ is encoded by the defective genome of the MAIDS virus.

CS016 and CS031 (Fig. 5, lanes 3 and 4). However, the $Pr65^{gag}$ was detected in all three RF/J cell lines (Fig. 5, lanes 2–4). Additional species of distinct molecular weight, possibly representing aberrant proteins, could also be detected (Fig. 5).

We also quantified virus production of these cells by evaluating the RT activity in their supernatant. The RT activity was strongly positive for the C57BL/6 cell line E2034 (>600,000 cpm) and weakly positive for the RF/J cell lines (20,000–50,000 cpm) (Table 2).

A summary on the characteristics of the cell lines is given in Table 2. Together, these data indicate the presence of replication-competent MuLV in all of these four cell lines, although helper-free stocks of the MAIDS defective virus was inoculated.

Cell surface phenotype of the malignant T-cell lines derived from diseased MAIDS mice

To better identify these malignant cells, they were characterized in more details by fluorocytometry, using a FACScan, and markers specific for T-cells (Thy-1, CD3, CD4, CD8), for B-cells (κ, μ, B220), and for macrophages (Mac-1). These data are shown in Fig. 6. The three cell lines established from the RF/J tumors were phenotypically immature T-cells ($Thy\text{-}1^+$, $CD3^-$, $CD4^+$, $CD8^+$). Interestingly, one additional cell line established from tumor CS016 after the fifth transplantation (T-5) was Thy 1.2^+, $CD4^+$, $CD8^+$, and $CD3^+$ (data not shown), indicating the differentiation of this tumor *in vivo* from $CD3^-$ to $CD3^+$. These three T cell lines were also Mac-1^+. Transcription of Mac-1 RNA was confirmed by Northern blot analysis, using Mac-1-specific probe, for cell-line CS016 (data not shown). The C57BL/6 T-cell line E2034 exhibited the phenotype of mature T-cell ($Thy\text{-}1^+$, $CD3^+$, $CD4^+$, $CD8^-$).

Altogether, these results showed that malignant T-cells, which can be established *in vitro* as cell lines, are present as rare clones in animals inoculated with helper-free stocks of the MAIDS defective virus.

DISCUSSION

In our groups of mice inoculated with helper-free stocks of the MAIDS defective virus, we found that T-cell lymphoma (detected by TcRβ gene rearrangement in enlarged lymphoid organs or by transplantation) was a relatively rare (~6%) phenomenon in C57BL/6 mice and may be more common (23%) in RF/J mice. These results contrast with previous observations in which replication-competent stocks LP-BM5 strain of the MAIDS defective virus were used. Klinken *et al.* (1988) reported clonal TcRβ rearrangements (very likely reflecting malignant T-cell expansion) in 35% of their MAIDS mice inoculated with the LP-BM5 MAIDS virus stocks. Kubo *et al.* (1992) also obtained transplantable T-cell lymphomas from C57BL/6 donor MAIDS mice at high frequency (71%), using (C57BL/6 × BALB/c) F1 nude recipient mice. Similarly, Tang *et al.* (1992) derived T-cell lymphomas in recipient SCID mice inoculated with cells from C57BL/6 MAIDS donor mice at a frequency of 25% (at 9 weeks

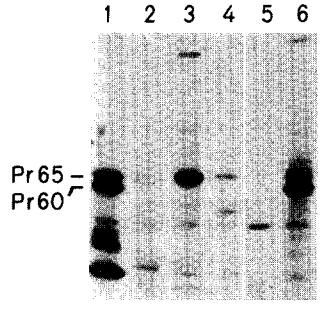

FIG. 5. Immunoprecipitation of the precursor gag proteins in cell lines established from MAIDS mice. Cells were from the established lines E20/34 (lane 1), E20/28 (lane 2), CS016 (lane 3), and CS031 (lane 4), from control noninfected NIH/3T3 (lane 5) or control infected $\Psi 2$ cells transfected with the MAIDS defective virus (lane 6). Cells were labeled with [^{35}S]methionine as described under Materials and Methods and the labeled proteins were immunoprecipitated with goat anti-gag p30 anti-serum. Immunoprecipitated proteins were separated by 5 to 20% SDS–PAGE. Note the $Pr65^{gag}$ and $Pr60^{gag}$ encoded by helper MuLV and by the defective MAIDS virus, respectively.

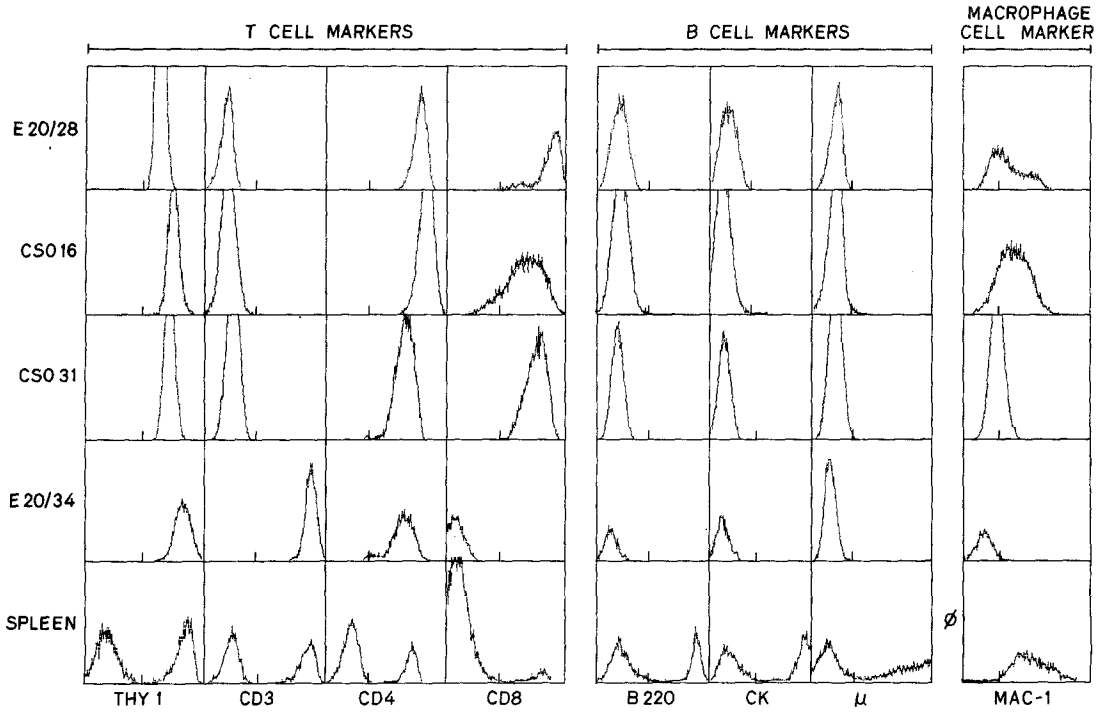

FIG. 6. Cell surface phenotype of established cell lines from MAIDS mice. Cells from the established line E20/28, CS016, CS031, E20/34, and from control C57BL/6 spleen or peritoneal adherent macrophages (ϕ) were stained with a panel of monoclonal antibodies specific for T cells (Thy 1.1 or 1.2, CD3, CD4, and CD8), B cells (B220, C_κ, μ), or macrophages (Mac-1). Cell surface stainings were carried out by using either a direct (Thy-1, CD3, CD4, CD8, μ) or an indirect (B200, C_κ, Mac-1) immunofluorescence assay. Stained cells were analyzed on a FACScan. At least 5000 cells are included in each fluorescence histogram. The vertical lines indicate the lower limits of the positivity, based on negative controls (cells unstained (direct labeling) or stained with only the FITC Rat anti-mouse IgG (indirect labeling)). Note that the RF/J cell lines (E20/28, CS016, CS031) are Thy1$^+$, CD3$^-$, CD4$^+$, CD8$^+$, Mac-1$^+$, while the C57BL/6 cell line (E20/34) is Thy-1$^+$, CD3$^+$, CD4$^+$, CD8$^-$, Mac-1$^-$.

p.i.), 40% (at 12 weeks p.i.), and 83% (at 31 or 32 weeks p.i.). The discrepancies between those studies and this one could be related to the age at which these mice were killed, to the environment in which they were maintained, to the recipient mice used, and more likely to the virus stocks inoculated. The crude LP-BM5 virus stocks used by these investigators (Klinken *et al.*, 1988; Kubo *et al.*, 1992; Tang *et al.*, 1992) contain a mixture of different helper MuLVs (ecotropic and mink-cell-focus-forming viruses) (Haas and Reshef, 1980; Hartley *et al.*, 1989; Chattopadhyay *et al.*, 1989, 1991). These helper MuLVs alone or pseudotyped with the MAIDS defective virus may contribute to the T-cell expansion observed by these investigators. In contrast, our stocks of the MAIDS defective virus were helper-free, thus significantly reducing virus reinfection and the contribution of the helper MuLV in T-cell transformation.

The apparent higher frequency of T-cell lymphomas detected in RF/J mice, as compared to C57BL/6 mice, with the same virus preparations, may not be significant because of the low number of tumors observed. If real, this difference is likely to reflect the genetic background of RF/J mice. These are known to develop a relatively high incidence of spontaneous thymic lymphomas (Gross, 1970; Goodenow and Lilly, 1984) and to be very susceptible to T-cell tumor development after irradiation or chemical treatment (Gross, 1970; Duran-Reynals *et al.*, 1978). Klinken *et al.* (1988) also detected a difference in the TcRβ clonal rearrangements in MAIDS lymphoid organs from two different strains of mice. In addition, the apparent different incidence of T cell lymphoma arising in C57BL/6 and RF/J MAIDS mice may reflect the presence of replicating endogenous MuLV in RF/J mice. We have indeed detected endogenous ecotropic MuLVs in all RF/J mice, but only rarely in C57BL/6 mice, inoculated with the helper-free stocks of the MAIDS defective virus (Fig. 2A). These MuLVs are likely to serve as helpers to rescue the MAIDS defective virus. In agreement with this interpretation, we observed that all recipient RF/J mice, but not the C57BL/6 mice, transplanted with grafted cells from syngeneic donor-diseased MAIDS mice developed the MAIDS syndrome. In addition, helper MuLVs were present in the three transplantable T-cell lymphomas and cell lines originating from RF/J mice. Unexpectedly, helper MuLVs were also detected in the T-cell lymphoma derived from a C57BL/6 mouse. These helper MuLVs are likely to be derived from endogenous MuLV sequences. Together, these results suggest that T-cell lymphoma develop in MAIDS mice only when helper MuLVs are present to allow reinfection of T-cells. These endogenous

helper MuLVs may contribute to T-cell transformation, although our data do not allow us to establish this causal role. However, not all helper MuLV may be of equal efficiency in contributing to T-cell transformation in MAIDS mice as none of the 16 mice inoculated with the helper-competent stocks of the MAIDS defective virus produced by the E20/34 T-cell line develop T-cell lymphoma, but all develop MAIDS.

In addition, the MAIDS defective viral genome itself may contribute to T-cell transformation *in vivo*. Indeed, the defective viral genome and its gene product $Pr60^{gag}$ was clearly detected in the cell line E20/34. Also, all the T-cell tumors derived by Kubo *et al.* (1992) and Tang *et al.* (1992) harbor the MAIDS defective genome. However, the specific MAIDS $Pr60^{gag}$ protein was not detected in two of four transformed RF/J T-cell lines, suggesting that the presence of this specific MAIDS viral protein may not be essential for the transformation of T-cells. More specific experiments will have to be designed to determine whether the defective virus is involved in T-cell transformation.

The four T-cell lines that we have established were phenotypically distinct depending on their origin. The tumor cells originating from the C57BL/6 mouse were mature $CD4^+$ $CD8^-$ T-cells, a phenotype similar to the tumors obtained by Tang *et al.* (1992), while those originating from RF/J mice were immature $CD4^+CD8^+$ T-cells. These RF/J cell lines were $CD3^-$ and only one had a rearranged TcRβ gene, indicating they were derived from early T-cell progenitors. All these three lines also expressed Mac-1, a macrophage/monocyte cell surface marker. This constitutes a particularly intriguing phenotype. A subpopulation of immature Mac-1^+ precursor T-cells is known to be present among murine bone marrow cells (Holmes *et al.*, 1988). The virus may have initially infected this population. Alternatively, the expression of the Mac-1 molecule may have been induced in these immature transformed T-cells after their infection. Our present data do not allow us to discriminate between both possibilities. All of our four cell lines were Thy-1^+. This finding contrasts with the Thy-1^- phenotype of the T-cell lymphomas derived by Kubo *et al.* (1992) from C57BL/6 MAIDS mice, after transplantation of their lymphoid tissues into nude mice. The different transplantation protocols or virus stocks used by both groups may explain the preferential growth of one type of T-cell lymphoma.

Interestingly, the transplantation protocol used here did not allow the growth of the infected B cells which constitute the majority of the infected cells in animals inoculated with helper-free stocks of the MAIDS defective virus (Huang *et al.*, 1991). Alternative experimental protocols will be needed to establish these cells *in vitro* early after their expansion *in vivo*.

In summary, our study shows that transformation of T-cells in two strains of mice inoculated with the MAIDS defective virus is a rare event whose frequency appears to depend on the presence of pathogenic helper virus in the viral stocks used or in mouse strains inoculated. T-cell lines can be established relatively easily out of these T-cell tumors, suggesting that these T-cell tumors are fairly aggressive. The availability of these T-cell lines would be useful to determine whether they have conserved some of the characteristics of the anergic T-cells *in vivo*.

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REFERENCES

ALT, F. N., ROSENBERG, S., LEWIS, S., THOMAS, E., and BALTIMORE, D. (1981). Organization and reorganization of immunoglobulin genes in A-MuLV-transformed cells: Rearrangements of heavy but not light chain genes. *Cell* **27,** 381–390.

ASTIER, T., GUILLEMAIN, B., LAIGRET, F., MAMOUN, R., and DUPLAN, J. F. (1982). Serological characterization of C-type retroviruses endogenous to the C57BL/6 mouse and isolated in tumours induced by radiation leukaemia virus (RadLV-Rs). *J. Gen. Virol.* **61,** 55–63.

AZIZ, D. C., HANNA, Z., and JOLICOEUR, P. (1989). Severe immunodeficiency disease induced by a defective murine leukaemia virus. *Nature* **338,** 505–508.

BILELLO, J. A., KORT, J. J., MACAULEY, C., FREDRICKSON, T. N., YETTER, R. A., and EISEMAN, J. L. (1992). ZDV delays but does not prevent the transmission of MAIDS by LP-BM5 MuLV-infected macrophage-monocytes. *J. AIDS* **5,** 571–576.

CACCIA, N., KRONENBERG, M., SAXE, D., HAARS, R., BRUNS, G. A. P., GOVERNMAN, J., MALISSEN, M., WILLARD, H., YOSHIKAI, Y., SIMON, M., HOOD, L., and MAK, T. (1984). The T cell receptor B chain genes are located on chromosome 6 in mice and chromosome 7 in humans. *Cell* **37,** 1091–1099.

CHATTOPADHYAY, S. K., LANDER, M. R., RANDS, E., and LOWY, D. R. (1980). Structure of endogenous murine leukemia virus DNA in mouse genomes. *Proc. Natl. Acad. Sci. USA* **77,** 5774–5778.

CHATTOPADHYAY, S. K., MORSE, I. I. I., MAKINO, M., RUSCETTI, S. K., and HARTLEY, H. W. (1989). Defective virus is associated with induction of murine retrovirus-induced immunodeficiency syndrome. *Proc. Natl. Acad. Sci. USA* **86,** 3862–3866.

CHATTOPADHYAY, S. K., SENGUPTA, D. N., FREDRICKSON, T. N., MORSE, I. I. I., and HARTLEY, J. W. (1991). Characteristics and contributions of defective, ecotropic, and mink cell focus-inducing viruses involved in a retrovirus-induced immunodeficiency syndrome of mice. *J. Virol.* **65,** 4232–4241.

CHEUNG, S. C., CHATTOPADHYAY, S. K., HARTLEY, J. W., MORSE, I. I. I., and PITHA, P. M. (1991). Aberrant expression of cytokine genes in peritoneal macrophages from mice infected with LP-BM5 MuLV, a murine model of AIDS. *J. Immunol.* **146,** 121–127.

CHOMCZYNSKI, P., and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.

DURAN-REYNALS, M. L., LILLY, F., BOSCH, A., and BLANK, K. J. (1978). The genetic basis of susceptibility to leukemia induction in mice by 3-methylcholanthrene applied percutaneously. *J. Exp. Med.* **147,** 459–469.

FEINBERG, A. P., and VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132,** 6–13.

FREDRICKSON, T. N., TANG, Y., CHATTOPADHYAY, S. K., MORSE, H. C., III, and HARTLEY, J. W. (1993). Retrovirus-induced lymphoproliferation as a model for developing diagnostic criteria for malignant lymphoma in mice. *Toxicol. Pathol.* **21,** 219–228. (Abstract)

GOODENOW, M. M., and LILLY, F. (1984). Expression of differentiation and murine leukemia virus antigens on cells of primary tumors and cell lines derived from chemically induced lymphomas of RF/J mice. *Proc. Natl. Acad. Sci. USA* **81,** 7612–7616.

GORSKA-FLIPOT, I., HUANG, M., CANTIN, M., RASSART, E., MASSÉ, G., and JOLICOEUR, P. (1992). U3 LTR-mediated induction of intracellular immunity by a murine retrovirus: A novel model of latency for retroviruses. *J. Virol.* **66,** 181–216.

GROSS, L. (1970). "Oncogenic viruses." *Pergamon, Elmsford, NY.*

GUILLEMAIN, B., ASTIER, T., MAMOUN, R., and DUPLAN, J. F. (1980). In vitro production and titration assays of B-tropic retroviruses isolated from C57BL mouse tumors induced by radiation leukemia virus (RadLV-Rs): Effect of dexamethasone. *Intervirology* **13,** 65–73.

HAAS, M., and RESHEF, T. (1980). Non-thymic malignant lymphomas induced in C57BL/6 mice by cloned dualtropic viruses isolated from hematopoietic stromal cell lines. *Europ. J. Cancer* **16,** 909–917.

HARTLEY, J. W., FREDRICKSON, T. N., YETTER, R. A., MAKINO, M., and MORSE, I. I. I. (1989). Retrovirus-induced murine acquired immunodeficiency syndrome: Natural history of infection and differing susceptibility of inbred mouse strains. *J. Virol.* **63,** 1223–1231.

HITOSHI, Y., OKADA, Y., SONODA, E., TOMINAGA, A., MAKINO, M., SUZUKI, K., KINOSHITA, J., KOMURO, K., MIZUOCHI, T., and TAKATSU, K. (1993). Delayed progression of a murine retrovirus-induced acquired immunodeficiency syndrome in X-linked immunodeficient mice. *J. Exp. Med.* **177,** 621–626.

HOLMES, K. L., LEE, J. S., and MORSE, I. I. I. (1988). Mac-1+ bone marrow cells include precursors of B cells and T cells. *Curr. Top. Microbiol. Immunol.* **141,** 19–26.

HUANG, M., and JOLICOEUR, P. (1990). Characterization of the gag/fusion protein encoded by the defective Duplan retrovirus inducing murine acquired immunodeficiency syndromes. *J. Virol.* **64,** 5764–5772.

HUANG, M., SIMARD, C., and JOLICOEUR, P. (1989). Immunodeficiency and clonal growth of target cells induced by helper-free defective retrovirus. *Science* **246,** 1614–1617.

HUANG, M., SIMARD, C., and JOLICOEUR, P. (1992). Susceptibility of inbred strains of mice to murine AIDS (MAIDS) correlates with target cell expansion and high expression of the defective MAIDS virus. *J. Virol.* **66,** 2398–2406.

HUANG, M., SIMARD, C., KAY, D. G., and JOLICOEUR, P. (1991). The majority of cells infected with the defective murine AIDS virus belong to the B-cell lineage. *J. Virol.* **65,** 6562–6571.

JOLICOEUR, P. (1991). The murine acquired immunodeficiency syndrome (MAIDS): An animal model to study the AIDS pathogenesis. *FASEB J.* **5,** 2398–2405.

KLINKEN, S. P., FREDRICKSON, T. N., HARTLEY, J. W., YETTER, R. A., and MORSE, I. I. I. (1988). Evolution of B cell lineage lymphomas in mice with a retrovirus-induced immunodeficiency syndrome, MAIDS. *J. Immunol.* **140,** 1123–1131.

KUBO, Y., NAKAGAWA, Y., KAKIMI, K., MATSUI, H., IWASHIRO, M., KURIBAYASHI, K., MASUDA, T., HIAI, H., HIRAMA, T., YANAGAWA, S. I., and ISHIMOTO, A. (1992). Presence of transplantable T-lymphoid cells in C57BL/6 mice infected with murine AIDS virus. *J. Virol.* **66,** 5691–5695.

LEGRAND, E., GUILLEMAIN, B., DALCULSI, R., and LAIGRET, F. (1982). Leukemogenic activity of B-ecotropic C-type retroviruses isolated from tumors induced by radiation leukemia virus (RadLV-Rs) in C57BL/6 mice. *Int. J. Cancer* **30,** 241–247.

LEWIS, S., ROSENBERG, N., ALT, F., and BALTIMORE, D. (1982). Continuing kappa-gene rearrangement in a cell line transformed by Abelson murine leukemia virus. *Cell* **30,** 807–816.

MAMOUN, R., GUILLEMAIN, B., ASTIER, T., PORTAIL, J. P., LEGRAND, E., and DUPLAN, J. F. (1978). Production et analyse d'un complexe viral dérivé d'un virus des radioleucoses de la souris C57BL. *Int. J. Cancer* **22,** 98–105.

MAX, E. E., MAIZEL, J. V., JR, and LEDER, P. (1981). The nucleotide sequence of a 5.5-kilobase DNA segment containing the mouse k immunoglobulin J and C region genes. *J. Biol. Chem.* **256,** 5116–5120.

MORSE, I. I. I., CHATTOPADHYAY, S. K., MAKINO, M., FREDRICKSON, T. N., HUGIN, A. W., and HARTLEY, J. W. (1992). Retrovirus-induced immunodeficiency in the mouse: MAIDS as a model for AIDS. *AIDS* **6,** 607–621.

MOSIER, D. E. (1986). Animal models for retrovirus-induced immunodeficiency disease. *Immunol. Invest.* **15,** 233–261.

PAQUETTE, Y., DOYON, L., LAPERRIÈRE, A., HANNA, Z., BALL, J., SEKALY, R. P., and JOLICOEUR, P. (1992). A viral Long Terminal Repeat expressed in CD4+CD8+ precursors is downregulated in mature peripheral CD4−CD8+ or CD4+CD8− T cells. *Mol. Cell. Biol.* **12,** 3522–3530.

POIRIER, Y., and JOLICOEUR, P. (1989). Distinct helper virus requirements for Abelson murine leukemia virus-induced pre-B- and T-cell lymphomas. *J. Virol.* **63,** 2088–2098.

SAVARD, P., DESGROSEILLERS, L., RASSART, E., POIRIER, Y., and JOLICOEUR, P. (1987). Important role of the long terminal repeat of the helper Moloney murine leukemia virus in Abelson virus-induced lymphoma. *J. Virol.* **61,** 3266–3275.

SIMARD, C., HUANG, M., and JOLICOEUR, P. (1994). Murine AIDS is initiated in the lymph nodes draining the site of inoculation, and the infected B cells influence T cells located at distance, in noninfected organs. *J. Virol.* **68,** 1903–1912.

TANG, Y., FREDRICKSON, T. N., CHATTOPADHYAY, S. K., HARTLEY, J. W., and MORSE, I. I. I. (1992). Lymphomas in mice with retrovirus-induced immunodeficiency. *Curr. Top. Microbiol. Immunol.* **182,** 395–398.